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A longevity protein, Lag2, interacts with SCF complex and regulates SCF function

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 26 March 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. I would first like to apologise for the length of time taken to get back to you with a decision. Your manuscript has now been seen by three referees, whose comments are enclosed below. As you will see, all three recognise the potential interest in your discovery of Lag2 as an interacting partner and regulator of the SCF complex. However, while referee 3 is more positive, reviewers 1 and 2 raise serious concerns as to both the mechanistic and physiological insight provided by your study. Currently, they find that the mechanism by which Lag2 regulates SCF complex rubylation remains incompletely understood, as do the downstream consequences on SCF function. In our assessment, these concerns preclude publication of your manuscript at this stage. Moreover, while the referees suggest a number of experiments that might allow you to address these criticisms, it is not clear to us that you will be able to resolve these issues. Even if you are able to do so, we feel that the amount of work required would go well beyond the scope of a normal revision.

In the light of these recommendations from three experts in the field, implicit in the comments attached, but also more explicitly stated in the confidential remarks to the editor, I am afraid we see little choice other than to reject your manuscript at this stage. Given the high number of submissions we receive, we can only consider those which receive an enthusiastic report from at least a majority of the referees upon initial review. However, given the interest expressed by the referees, we would be willing to consider a resubmission of your study, should you be able to provide significantly greater mechanistic insight and clarity. I should state that this would have to be judged as a resubmission, rather than a revision, and would be assessed again, perhaps involving new reviewers,

and also in the light of any literature published in the intervening period.

I am sorry we can not be more positive on this occasion, but we hope that you find the referees' comments helpful when revising the manuscript for future submission here or elsewhere.

REFEREE REPORTS

Referee #1 (Remarks to the Author):

A longevity protein, Lag2, interacts with SCF complex and regulates SCF function

This manuscript identifies longevity factor, Lag2, as a Cdc53 interacting protein. Lag2 binding is specific to unrubylated Cdc53, and requires both N- and C-terminal of Cdc53. Lag2 overexpression reduces the rubylation level of Cdc53, whereas lag2 deletion results in accumulation of rubylated Cdc53. Lag2 inhibits Cdc53 rubylation in vitro as well as negatively regulates substrate ubiquitination by SCF. Finally, lag2 deletion in combination with dcn1 Δ results in slow growth phenotype of yeast cells.

Even though this manuscript documents Lag2 as a novel SCF interacting protein that affects the rubylation levels of Cdc53, it fails to provide a thorough insight into how it plays a role in regulating the activity of SCF complex. Also, the authors need to talk more about Lag2 and show how it inhibits rubylation rather than speculating that Lag2 works by competing with Dcn1. The authors see either a modest phenotypic effect or small changes in SCF activity; however, they claim "Lag2 plays a fundamental role in regulating ...." in the abstract.

In Figure 1C, Cdc53 in rub1 Δ is mostly unmodified whereas in jab1 Δ is mostly rubylated. However, in the HA-Lag2 immunoprecipitated lanes (Figure 1C), the authors see same levels of unmodified Cdc53. They need to explain this observation.

What is the basis for Lag2's specific interaction with the unmodified form of Cdc53 and not the rubylated form?

Interaction with Lag2 requires both the N- and C-terminal of Cdc53. The authors should elaborate on this observation, since these 2 regions are separated by more than 50 Å. Also, does making specific mutation in Cdc53 near the neddylation site that abolishes Dcn1 interaction, affects Cdc53 rubylation in lag2 deletion?

Why are the Cdc53 rubylation levels so different in WT cell (lane 1) of figure 3A and 3B? Also, the authors should quantify there western analysis since the differences are modest. For instance, unmodified Cdc53 in Lane 4 of the long exposure (Figure 3A) could be due to bleed over from the adjacent lane.

Does Lag2 decreases rubylation rate by competing for Dcn1 binding site or by affecting some other step of the reaction? Also, how efficiently does Lag2 inhibits Cdc53 (Δ1-133) rubylation level?

What is the rate of rubylation in a rub1 Δdcn1 Δlag2 Δ strain at high E2 concentration? Based on the proposed model, the rubylation level should be greater than in rub1 Δdcn1 Δ (figure 4B).

The levels of rubylated Cdc53 are different in lag2 Δdcn1 Δ and lag2 Δdcn1 Δjab1 Δ (fig 3B), even though both show slow growth phenotype (fig 5). However, neddylation levels of dcn1 Δ and lag2 Δdcn1 Δ are same, but they show different growth phenotypes. Hence, Lag2's role in regulating Cdc53 rubylation level does not directly correlate with cell growth. The authors should explain this discrepancy.

How does the rate of Sic1 ubiquitination with increase in Lag2, correlates with the Cdc53 rubylation level?

Referee #2 (Remarks to the Author):

Liu and colleagues describe a novel interactor (Lag2) of SCF ligases in budding yeast. They identified Lag2 in a 2-hybride screen using F-box subunits and went on to show that it specifically interacts with Cdc53/Cul1 but not other cullins in budding yeast. The authors suggest that the interaction is specific for non-rubylated Cdc53, although I am not convinced by the evidence (see below). The authors do a set of pretty convincing in vitro assays that demonstrate that Lag2 counteracts rubylation of Cdc53. The mechanism of how Lag2 counteracts rubylation is not clear but the authors speculate that binding of Lag2 might block rubylation. The authors also provide a panel of genetic interaction experiments, which I found confusing and somewhat inconsistent with the models put forward. Together, this manuscript reports a novel interactor of SCF ubiquitin ligases that regulates Cul1 rubylation and affects SCF function. This is an important subject of broad interest and there is certainly something to this manuscript that makes it interesting enough for publication in EMBO. Particularly the in vitro results indicate a regulatory role of Lag2 in Cul1 rubylation. However, I do not support publication of the manuscript in the current stage, because (i) I believe the manuscript does not go far enough to provide mechanistic insight. Some of the conclusions drawn are not very well supported by the results. (ii) There was very little effort to show an effect of Lag2 misregulation on the stability of SCF substrates. The only experiment in this direction is presented in figure 7D, which I think is not very well planned as it cannot distinguish between regulation of protein stability (as the authors infer) and regulation at the transcription or translation level. Overall, I think these points can be addressed with additional experiments.

Specific comments:
Major comments:
1) Specific binding to non-rubylated Cdc53: The authors conclude that Lag2 binds specifically to non-rubylated Cdc53. This is mainly based on IP experiments particularly shown in figure 1C, where Lag2 is immunopurified and the co-purified Cdc53 is in its non-rubylated form. The point that makes me think there is something else going on here is that in jab1 mutants almost all the Cdc53 is in its rubylated form, but the IP shows equivalent amounts of NON-rubylated Cdc53 co-purified with Lag2 when compared to a rub1 deletion mutant where all the Cdc53 is in its non-rubylated form. One possibility is that there was significant derubylation going on during the IP incubation. This could also result from derubylation activity associated with Lag2 itself or binding partners. An alternative explanation is that Lag2 binds only a tiny fraction of Cdc53. In any case, the authors need to analyze lysates in these experiments that were mock-incubated in parallel with the IP incubation to exclude that they lose the Rub1 modification during the IP incubation. In general, binding to non-rubylated Cdc53 should be tested more rigorously before drawing this conclusion.

2) The authors did not demonstrate direct interaction between Lag2 and Cdc53. They mention in the discussion section that they find binding between Lag2 and Cdc53 using bacterially expressed recombinant proteins. This is an important result and should be part of Figure 1.

3) There are some nice in vitro experiments, but almost no analysis of the effect of Lag2 deletion or overexpression on substrate stability in vivo. As mentioned above, the experiment shown in figure 7D testing Aah1 stability by looking at steady-state Aah1 levels is not appropriate. The authors show an effect on Sic1 ubiquitination in vitro, so I think at a minimum they need to test the effect of lag2 deletion and Lag2 overexpression on Sic1 degradation. This can be done by using an alpha factor release approach combined with Gal-Sic1 shut-off as has been described (for example: Petroski and Deshaies, Mol. Cell 2003). I also think the authors should look at Cln2 or Cln1 stability in both lag2 deletion strains and Lag2 overexpression conditions. This should be done by pulse-chase or Gal-shut off approaches to directly measure protein turn-over. These two well-established SCF substrates will demonstrate the role of Lag2 on substrate ubiquitination/degradation in vivo.

4) The genetic results are somewhat confusing. This might be because this part was particularly hard to follow in the text and would benefit by clarification. Many genetic interactions were carefully tested and reported, but I think to make them significant it will be important to show that the genetic interactions between SCF components and lag2 (particularly overexpression) depends on the rubylation pathway? For example, Lag2 overexpression should not lead to phenotypic enhancement

of a rub1delta skp1-11 double mutant if the observed genetics is a reflection of a role of Lag2 in rubylation.

Minor comments:
1) Figure 5B and C. Mutant phenotypes are scored as colony size. I assume the small colony size is a reflection of an increased doubling time. I think measuring the doubling time would result in a more accurate/convincing expression of the phenotypic defect.

2) The manuscript was quite hard to read and many sections could be clarified.

Referee #3 (Remarks to the Author):

In this manuscript, Liu et al. reported their identification of a novel SCF interacting protein, Lag2, in S. cerevisiae, which is reminiscent of CAND1 in mammalian cells. Although most of the regulatory factors of the SCF ubiquitin ligase complex, such as Rub1/Nedd8 and Jab1/CSN5, are conserved from yeast to humans, the yeast version of CAND1 has been missing due to the lack of any yeast ORF sharing recognizable sequence homology with human CAND1. The Lag2 protein, which is not homologous to CAND1 at the sequence level, seems to have several common functional features of CAND1. Interestingly, the two also shows substantial differences in other aspects. Overall, the paper identified a new regulator of yeast SCF and started to address its functional roles with a battery of genetic and biochemical approaches. Although many mechanistic questions remain to be answer, the findings do advance the field and shall be of interests to the general readers of EMBO J. This reviewer supports its publication after some revisions and clarification are made.

For Figure1C, please check whether Lag2 can co-IP known F-box proteins such as Cdc4 and Met30. This is important as other aspects of Lag2 implicates that it is a functional analogue of CAND1 in yeast, at least for Cdc53. In order to support or disapprove such a conclusion, it is essential to know whether Lag2 blocks SCF assembly or not. The authors show that Lag2 does not block Skp1-Cdc53 interaction and some F-box proteins can pull out Lag2 by Y2H. Does that mean that Lag2 lacks perhaps the most important known function of CAND1, which is to inhibit the assembly of SCF? Either way, this issue should be fully addressed.

It is disappointing that the authors cannot pin down the mechanism underlying the antagonistic actions of Dcn1 and Lag2 through binding assays. The authors mention that they failed to detect the co-IP of Dcn1 and Cdc53, therefore, they could not test whether Lag2 can compete with Dcn1 for Cdc53 binding. How about checking Lag2-Cdc53 interaction (Figure 1) in the presence of either Dcn1 overexpression or increasing amounts of recombinant Dcn1?

The authors emphasize on the possible role of Lag2 in inhibiting Cdc53 rubylation. Many experiments were designed to support this concept. It is not clear why it could not be the other way around, i.e., rubylation inhibits Lag2-Cdc53 interaction. These two scenarios will assign the actions of most cullin cycle regulators in completely opposite directions. Please discuss why one is favored over the other.

There are a few grammatical mistakes and typos throughout the text. For example, last sentence on page 6 should read "Combined with metalloprotease Csn5/Jab1, how all these regulators balance neddylation and deneddylation of .....". In the last paragraph on Page 16, "exacts" should be "extracts".

Authors' Response 01 June 2009

Response to Referee 3:

1. *Whether Lag2 co-immunoprecipitated known F-box protein should be tested, which is important to distinguish Lag2 from CAND1.*

We performed the binding experiment *in vivo* (new Figure 1C) and observed that endogenous Lag2 interacted with all the components of SCF^{Cdc4} - Cdc53, Skp1, Rbx1 and F-box protein Cdc4. Similarly, we conducted the binding experiment *in vitro*, with recombinant Lag2 purified from E. coli and recombinant SCF^{Cdc4} complex from insect cells, and further confirmed the association between Lag2 and all the subunits of SCF^{Cdc4} (new Figure 1D). We thus conclude that Lag2 lacks perhaps the most important known function of CAND1, which inhibits the assembly of SCF complex. We have clarified this issue in the modified manuscript (page 10, lines 2 - 9).

2. *Does Dcn1 overexpression affect the Lag2-Cdc53 interaction through binding assays, which may explain the antagonistic actions of Dcn1 and Lag2?*

Although we failed to detect the co-immunoprecipitation of either Dcn1 and Cdc53 or Lag2 and Dcn1 *in vivo*, previous reports (Kurz et al., 2005, Nature, 435, 1257-1261, Kurz et al., 2008, Mol Cell, 29, 23-35) show that Dcn1 interacts with Cdc53 in *in vitro* binding assay and yeast two-hybrid assay. We reasoned that Dcn1 may bind to Cdc53 with low affinity in physiological condition. So, we examined whether deletion or overexpression of Dcn1 affected the Lag2-Cdc53 interaction or not. We found that Dcn1 inhibited the binding of Lag2 to Cdc53, which suggests that Lag2 can function antagonistic to Dcn1. We have clarified this issue in the modified manuscript (new Figure 3, page 15, line 6 - page 16, line 14).

3. *Two scenarios, one is that Lag2 inhibits the rubylation of Cdc53, the other is that rubylation inhibits Lag2-Cdc53 interaction should be appropriately discussed in the text.*

We have discussed the two scenarios in the Discussion session in the modified manuscript (page 24, line 17 - page25, line 14.)

4. *Grammatical mistakes and typos should be corrected.*

We have asked Nature Publishing Group Language Editing to do the proof-reading of our manuscript.

Response to Referee 2:

Major comments:
1. *Does Lag2 specifically bind to non-rubylated Cdc53? Derubylation during the immunoprecipitation process should be excluded.*

We performed *in vivo* binding assay between Lag2 and Cdc53 with known F-box protein Saf1 as control and we found that Lag2 reproducibly interacted with non-rubylated Cdc53 in physiological condition (new Figure 1E). To exclude the possibility that derubylation occurred during immunoprecipitation, we compared the status of Cdc53 in the yeast cell extracts before and after antibody incubation (new Figure 1F). No derubylation of Cdc53 was detected in the process of immunoprecipitation. Thus, we conclude that Lag2 specifically binds to non-rubylated Cdc53. We have clarified this issue in the modified manuscript (page 10, line 10 - page 11, line 5).

2. *Direct interaction of Lag2 to Cdc53 should be demonstrated.*

We conducted the *in vitro* binding assay between recombinant Lag2 and recombinant SCF^{Cdc4} purified from *E. coli* and insect cells, respectively (new Figure 1D). We observed that Lag2 interacted with Cdc53, Cdc4, Skp1 and Rbx1. Together with the *in vivo* binding data, we conclude that Lag2 binds to all the subunits of SCF complex, which is different from CAND1 that associates with Cul1-Rbx1 module, exclusively of Skp1, F-box protein. We have clarified this point in the modified manuscript (page 10, lines 4 - 9).

3. *The role of Lag2 on SCF substrate degradation in vivo should be further demonstrated.*

In the previous manuscript, we detected that deletion of *lag2* increased the abundance of Aah1, substrate of SCF^{Saf1}. Because we tested the expression of Aah1 in the late logarithmic phase, we

could not measure Aah1 turnover. Therefore, to further investigate the effects of Lag2 on SCF function, we performed Sic1 turnover experiment *in vivo* and observed that deletion of *lag2* induced a marked decrease of the stability of Sic1 (new Figure 6E). Together with the *in vitro* assay, we conclude that Lag2 negatively regulates the activity of SCF E3 ubiquitin ligase. We have clarified this point in the modified manuscript (page 19, line 15 – page 20, line 11).

4. *Is the genetic interaction between SCF components and Lag2 dependent on the rubylation pathway?*

Based on the observation that Lag2 overexpression suppresses the *skp1-11* ts cell growth, the referee concerned whether the overexpression of Lag2 affects *skp1-11* cell growth or not, in the absence of Rub1 (rubylation pathway). In the previous manuscript, we showed that deletion of *rub1* obviously suppressed the *cdc34-2* ts cell viability, while the additional deletion of *lag2* partially rescued the cell viability (Figure 7A, new Figure 6A). These data suggest that Lag2 also can affect SCF function independent of rubylation pathway. With this notion, we have not performed additional experiment, using *skp1-11* ts cell.

Minor comments:
1. *Is the small colony size a reflection of an increased doubling time?*

We agreed with this concern. We reproducibly confirmed the growth defects exerted by double deletion of *lag2* and *dcn1*, or triple deletion of *lag2*, *dcn1* and *jab1* in plate.

2. *Manuscript should be clarified for easy reading.*

We have asked Nature Publishing Group Language Editing to do the proof-reading of our manuscript.

Response to Referee 1:

1. *Why does Lag2 co-immunoprecipitate same level of unmodified Cdc53 from yeast cell extracts with different status of Cdc53?*

In the previous manuscript, we mainly concerned that Lag2 specifically bound to one unrubylated Cdc53 band. According to the comment of referee, we carefully performed the *in vivo* binding experiments with the same amount of yeast cell lysates (new Figure 3B, 3C). We observed that the interaction of Lag2 to Cdc53 was clearly increased by the deletion of *rub1*, compared to wild type cells. We have clarified this issue in the modified manuscript (page 15, line 16 – page 16, line 14).

2. *What is the basis for Lag2's specific interaction with the unmodified form of Cdc53 and not the rubylated form?*

We performed *in vivo* binding assay between Lag2 and Cdc53 with known F-box protein Saf1 as control and we found that Lag2 reproducibly interacted with non-rubylated Cdc53 in physiological condition (new Figure 1E). To exclude the possibility that derubylation occurred during immunoprecipitation, we compared the status of Cdc53 in the yeast cell extracts before and after antibody incubation (new Figure 1F). No derubylation of Cdc53 was detected in the process of immunoprecipitation. Thus, we conclude that Lag2 specifically binds to non-rubylated Cdc53. We have clarified this issue in the modified manuscript (page 10, line 10 - page 11, line 5).

3-1. *How is it explained that interaction with Lag2 requires both the N- and C-terminal of Cdc53?*

Molecular weights of Lag2 and Cdc53 are 76 kDa and 94 kDa, respectively. Different from Dcn1 (32 kDa), Lag2 has the similar molecular weight to Cdc53. It is possible that both N- and C-terminal of Cdc53 are required for the interaction of Lag2 to Cdc53.

During our further exploration for the regulatory mechanism of Lag2, we found more important mechanism exerted by Lag2 on SCF complex. We decided to remove the binding data of Lag2 and Cdc53 mutants in the modified manuscript.

3-2. *Does making specific mutation in Cdc53 near the neddylation site that abolishes Dcn1 interaction, affects Cdc53 rubylation in lag2 deletion?*

Cdc53 mutant that abolished Dcn1 interaction should show similar rubylation status of Cdc53 to the single *dcn1* deletion strain. Our data (Figure 3B, new Figure 2B) show that no detectable difference in status of Cdc53 was detected in *dcn1* single mutant and *lag2 dcn1* double mutant. With this notion, we have not performed further experiment.

4. *Why are the Cdc53 rubylation levels so different in wild type cells of Figure 3A and 3B? Is the more unmodified Cdc53 in Lane 4 of the long exposure (Figure 3A) due to the bleed over from the adjacent lane?*

In the previous manuscript, to clearly show the effect of Lag2 overexpression and deletion, respectively, we used different exposure time to check the status of Cdc53 (Figure 3A, 3B). Considering the comment of referee, we performed the Lag2 overexpression experiment with the similar exposure time to Lag2 deletion assay and we confirmed that overexpression of Lag2 increased unrubylated Cdc53 in wild type cells (new Figure 2A). And in the previous manuscript, to test whether Lag2 functions independent of Dcn1 and Jab1 or not, we detected the status of Cdc53 with overexpression of Lag2 in the single deletion of *jab1* or double deletion of *dcn1* and *jab1*. We observed that in the absence of Dcn1 and Jab1, overexpression of Lag2 clearly increased the unrubylated Cdc53. But in *jab1* deletion yeast strain, as referee pointed out, we could not detect the obvious difference of state of Cdc53 with overexpression of Lag2 with short exposure time. Thus, in the modified manuscript, we just use the double deletion of *dcn1* and *jab1* (new Figure 2A). We have clarified this issue in the modified manuscript (page 12, lines 5 - 13).

5-1. *Does Lag2 decreases rubylation rate by competing for Dcn1 binding site or by affecting some other step of the reaction?*

Although we failed to detect the coimmunoprecipitation of either Dcn1 and Cdc53 or Lag2 and Dcn1 *in vivo*, previous reports (Kurz et al., 2005, Nature, 435, 1257-1261, Kurz et al., 2008, Mol Cell, 29, 23-35) show that Dcn1 interacts with Cdc53 in *in vitro* binding assay and yeast two-hybrid assay. We reasoned that Dcn1 may bind to Cdc53 with low affinity in physiological condition. So, we examined whether deletion or overexpression of Dcn1 affected the Lag2-Cdc53 interaction or not. We found that Dcn1 inhibited the binding of Lag2 to Cdc53, which suggests that Lag2 can function antagonistic to Dcn1. We have clarified this issue in the modified manuscript (new Figure 3, page 15, line 6 - page 16, line 14).

5-2. *Also, how efficiently does Lag2 inhibits Cdc53 (Δ1-133) rubylation level?*

As we mentioned in 3-1, we have not performed additional experiment.

6. *What is the rate of rubylation in a triple deletion of rub1, lag2, dcn1, compared to a double deletion of rub1, dcn1 in high E2 concentration?*

We performed the *in vitro* rubylation experiment with four yeast strains: the *rub1Δ* single mutant, *rub1Δ lag2Δ* double mutant, *rub1Δ dcn1Δ* double mutant, and *rub1Δ lag2Δ dcn1Δ* triple mutant in high E2 concentration. In the previous manuscript, we just showed the results of the first three strains. In the modified manuscript, we added the result of the triple mutant (new Figure 2D). Consistent of the prediction of referee, the rubylation level in *rub1Δ lag2Δ dcn1Δ* triple mutant was greater than in *rub1Δ dcn1Δ* double mutant. We have clarified this point in the modified manuscript (page 13, line 17 - page 14, line 6).

7. *Lag2's role in regulating Cdc53 rubylation level does not directly correlate with cell growth. This discrepancy should be explained.*

As referee mentioned, we also noticed that Cdc53 rubylation level did not directly correlate with cell growth. However, in the previous manuscript, we showed that deletion of *rub1* obviously deduced the *cdc34-2* ts cell viability, while the additional deletion of *lag2* partially rescued the cell viability (Figure 7A, new Figure 6A), which suggests that Lag2 also can affect SCF function independent of rubylation pathway. Together with biochemical experiment, we propose that Lag2 regulates the SCF

complex by controlling its rubylation cycle and its ubiquitin ligase activities, in turn, influences cell growth.

8. *How does the rate of Sic1 ubiquitination with increase in Lag2, correlates with the Cdc53 rubylation level?*

In *in vitro* ubiquitination assay, Cdc53 is also autoubiquitinated by SCF complex. So, it is difficult to distinguish the ubiquitinated or rubylated Cdc53. With this notion, we have not performed related experiment.

2nd Editorial Decision 24 June 2009

Thank you for your resubmission of your manuscript for consideration by the EMBO Journal. I sent it back to two of the original referees (#s 1&2), and have now received their reports. As you will see, both referees appreciate that you have made significant improvements to your study, and are broadly supportive of publication. However, it is clear that there are still a number of major concerns, which would need to be addressed before we are able to consider publication. I will not go through all the details here, but I would in particular like to draw your attention to the comments of referee 1 regarding inconsistencies between the biochemical and genetic results - it would be important to try and resolve these issues in your revised manuscript.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of both reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1 (Remarks to the Author):

A longevity protein, Lag2, interacts with SCF complex and regulates SCF function

This manuscript identifies a novel SCF interacting protein, Lag2 that shares several features of human CAND1. Lag2 is a longevity factor that specifically interacts with the unrubylated form of Cdc53. Lag2 over-expression reduces the rubylation level of Cdc53, whereas lag2 deletion results in accumulation of rubylated Cdc53. In vitro, Lag2 inhibits Cdc53 rubylation in competition with Dcn1, which is important for cell growth. Finally, Lag2 negatively regulates substrate ubiquitination by SCF by possibly disrupting Cdc34-SCF interaction. Overall, the authors identify a novel SCF interacting protein and show that it affects the Cdc53 rubylation levels. Although the paper in its current form fails to answer all the mechanistic insight, it does document a key missing regulator of SCF rubylation cycle. These findings are potentially exciting to the general reader.

Specific comments:

The authors show in vivo and in vitro binding between Lag2 and SCFCdc4 complex (fig 1C and 1D). Based on this they propose that Lag2 bind to all the components of SCF complex. Lag2 can still bind SCF via Cdc53 similar to CAND1, but lacks the ability to displace Skp1-F-box from the cullin. The authors need clarify their conclusion based on their observation.

There is no correlation between the Cdc53 rubylation levels (fig 2B) and the growth phenotype (fig

4A) in various mutants. For instance, dcn1 null and lag2 dcn1 null have similar Cdc53 rubylation level, yet different growth phenotype; whereas lag2 jab1 null and dcn1 jab1 null have same growth phenotype but different Cdc53 rubylation level. Therefore, the growth defects observed with lag2 mutants is not likely due to the Cdc53 rubylation level. The authors need to explain this discrepancy.

In fig 6B, higher Sic1 ubiquitination and in fig 6C, lower Sic1 ubiquitination could be due to altered Cdc34-SCF interaction or due to altered Cdc53 rubylation levels. The cause of this difference needs to be established. Also, is the inhibitory effect of Lag2 or stimulatory effect of lag2 deletion also seen at higher Cdc34 concentrations?

Biochemical analysis (fig 7) indicates that Lag2 inhibits association of Cdc34 to SCF. If this were true, lag2 deletion will rescue Cdc34 binding to SCF, resulting in suppression of growth defect in cdc34 mutants. This is consistent with the genetic analysis where they see weak suppression in cdc34-2 rub1 null strain (fig 6A). However, additional genetic experiments (fig 5) show that lag2 deletion is synthetic with cdc34-2 ts in inducing growth defect. This is inconsistent with the biochemical model and the authors need to explain the discrepancy.

Referee #2 (Remarks to the Author):

I reviewed the earlier version of this manuscript by Liu and colleagues and by the editors request I will treat this new version as a new submission.
I felt that the manuscript is significantly improved. The authors (i) identified Lag2 as an interactor with SCF complexes, (ii) show that Lag2 preferentially interacts with non-rubylated Cdc53, (iii) that Lag2 counteracts rubylation of Cdc53, (iv) show that Lag2 interferes with binding of Cdc34 to the SCF complex. The authors also show extensive genetic analysis, which generally shows only mild effects of LAG2 deletion or overexpression and collectively the genetic results are somewhat confusing given that most of the manuscript studies the effect of Lag2 on Cdc53 rubylation. However, as the authors indicate the results are consistent with an additional function of Lag2 in modulating Cdc34 binding to SCF, as shown in the new figure 7. This new experiment, showing reduced binding of Cdc53 to Cdc34 in the presence of Lag2, is very interesting. In fact this seems to be an indication that Lag2 might be the molecular component that links Cdc34 binding to the rubylation status of Cdc53 such that Lag2 binds to non-rubylated Cdc53 and reduces binding of Cdc34. Rubylation prevents binding of Lag2 and increases binding of Cdc34. I think most of the results of Lag2 on Cdc53 rubylation could be secondary effects and could be explained by the physical interaction of Lag2 with non-rubylated Cdc53. The authors might have a good reason to exclude such a role for Lag2 in linking rubylation to Cdc34 binding, but this is not clear from the experiments presented in this manuscript. In any case, the new experiment (fig. 7) seems to me a significant addition and if the authors can strengthen these findings (see below) and address the other points below I support publication in EMBO Journal.

1) The authors show that Lag2 prevents Cdc53 binding to Gst-Cdc34 (fig. 7). The authors need to show in a similar experiment whether this effect is only seen for non-rubylated Cdc53 or also for rubylated Cdc53. This should be fairly easy as the authors have recombinant SCF-Cdc4 with a mixture of rubylated and non-rubylated Cdc53 (Fig. 1D).

2) Fig. 7: The authors need to show the effect of Lag2 on Rbx1 binding to Cdc53

3) Fig. 6 D and E: Both results are presented as proof for Lag2 reducing degradation of SCF substrates. As mentioned in my first review, reduced Aah1 steady state levels are not proof for increased protein degradation. At a minimum the RNA data need to be included. But even then, how can the authors exclude that lag2 deletion affects entry into stationary phase. Similar the Sic1 experiment wasn't done in a way that unambiguously measures Sic1 degradation. Sic is quite stable in many phases of the cell cycle. Sic1 degradation should be measured in cells released from an alpha-factor synchrony combined with GAL-shut-off (for example: Petroski and Deshaies, Mol. Cell 2003). I also think that Cln1 or Cln2 degradation needs to be analyzed, because their degradation is not affected by cell cycle position and can be easily measured.

4) Fig. 4B and C: I still think it is not adequate to express the growth defect as "relative colony size".

Growth curves should be shown instead. The reduced colony size could also, for example, be a reflection of delayed recovery from the liquid-to-solid medium shift. In addition, it is important to include a rub1 lag2 dcn1 triple mutant to test whether the growth defect depends on rubylation.

Minor comments:
1. Page 10: "...Lag2 bound to all the components of SCF complex...". I think this is somewhat misleading because the experiment cannot distinguish between binding to one single component and binding to several components.

2. Page 12: "Overexpression of Lag2 dramatically increased...(Fig. 2A)." This is overstated, there is a minimal effect.

1st Revision - Authors' Response 27 July 2009

Response to Referee 1:

1. The authors show in vivo and in vitro binding between Lag2 and SCFCdc4 complex (fig 1C and 1D). Based on this they propose that Lag2 bind to all the components of SCF complex. Lag2 can still bind SCF via Cdc53 similar to CAND1, but lacks the ability to displace Skp1-F-box from the cullin. The authors need clarify their conclusion based on their observation

First, we identified Lag2 in yeast two-hybrid assay (Figure 1A). Specifically, Lag2 interacted with SCF type F-box proteins, rather than non-SCF type F-box protein. It suggested that Lag2 might bind to F-box protein through Cdc53. This notion was confirmed in the followed two-hybrid assay between Lag2 and Cdc53 (Figure 1B). Then, we demonstrated that Lag2 interacted with SCF complex in physiological condition (Figure 1C). Similarly, we detected the interaction of Lag2 and SCF complex *in vitro* (Figure 1D). Based on these findings, we conclude that Lag2 bind to SCF complex. Besides, Lag2 only interacted with the unrubylated Cdc53 *in vivo* (Figure 1C, E, G). And, Lag2 inhibited Rub1 conjugation to Cdc53 (Figure 2). Thus, we conceive that Lag2 partially possesses some CAND1-like functions, even if Lag2 lacks the ability to displace Skp1-F-box protein from the cullin protein. We have clarified this issue in the Discussion of the revised manuscript (page 26, line 18 - page 27, line 13).

2. There is no correlation between the Cdc53 rubylation levels (fig 2B) and the growth phenotype (fig 4A) in various mutants. For instance, dcn1 null and lag2 dcn1 null have similar Cdc53 rubylation level, yet different growth phenotype; whereas lag2 jab1 null and dcn1 jab1 null have same growth phenotype but different Cdc53 rubylation level. Therefore, the growth defects observed with lag2 mutants is not likely due to the Cdc53 rubylation level. The authors need to explain this discrepancy.

In the preparation for revised manuscript, we further examined cell growth in liquid medium (Figure 4D). Single mutant of *lag2*, *dcn1*, *jab1* or *rub1*, did not display any significant growth effect. However, double deletion of *lag2* and *rub1* showed similar growth defect to that of double deletion of *lag2* and *dcn1*. Triple deletion of *lag2*, *dcn1* and *rub1* did not show further growth effect, compared to the double deletion of lag2 and rub1. Additional deletion of *jab1* in this triple mutant failed to affect the cell growth. These results imply that the loss of Lag2 function, together with the defect of rubylation to Cdc53 results in the retardation of cell growth. We have clarified this issue in the revised manuscript (page 17, line 13 - page 18, line 4).

3. In fig 6B, higher Sic1 ubiquitination and in fig 6C, lower Sic1 ubiquitination could be due to altered Cdc34-SCF interaction or due to altered Cdc53 rubylation levels. The cause of this difference needs to be established. Also, is the inhibitory effect of Lag2 or stimulatory effect of lag2 deletion also seen at higher Cdc34 concentrations?

In the Sic1 ubiquitylation assay *in vitro*, we examined the rubylation level of Cdc53 and observed the rubylated Cdc53 did not change with the increased recombinant Lag2 (Figure 6C). Next, we tested the effect of Lag2 at higher Cdc34 concentration. In the high E2 condition, the inhibitory effect of Lag2 on Sic1 ubiquitylation clearly decreased (Figure 6D). In Figure 7A, we have already shown that Lag2 inhibited the interaction between Cdc34 and SCF complex. Combined these observations, we conclude that Lag2 suppresses Sic1 ubiquitylation *in vitro* by competing with Cdc34 for binding to SCF complex, instead of affecting Cdc53 rubylation level. We have clarified this issue in the revised manuscript (page 20, lines 6 - 13).

4. Biochemical analysis (fig 7) indicates that Lag2 inhibits association of Cdc34 to SCF. If this were true, lag2 deletion will rescue Cdc34 binding to SCF, resulting in suppression of growth defect in cdc34 mutants. This is consistent with the genetic analysis where they see weak suppression in cdc34-2 rub1 null strain (fig 6A). However, additional genetic experiments (fig 5) show that lag2 deletion is synthetic with cdc34-2 ts in inducing growth defect. This is inconsistent with the biochemical model and the authors need to explain the discrepancy.

In the presence of Rub1, Lag2 affects SCF E3 ligase activity by directly inhibiting the interaction of Cdc34 with SCF complex, and by regulating rubylation cycle which indirectly modulating SCF function. In the absence of Rub1, Lag2 controls SCF E3 activity by the direct interference to the association between Cdc34 and SCF complex. Based on these data, we considered that deletion of *lag2* affected *cdc34-2* ts cell viability in different way in the presence or absence of Rub1. We have clarified this issue in the Discussion of revised manuscript (page 25, line 11- page 26, line 1).

Response to Referee 2:

Major comments:
1. The authors show that Lag2 prevents Cdc53 binding to Gst-Cdc34 (fig. 7). The authors need to show in a similar experiment whether this effect is only seen for non-rubylated Cdc53 or also for rubylated Cdc53. This should be fairly easy as the authors have recombinant SCF-Cdc4 with a mixture of rubylated and non-rubylated Cdc53 (Fig. 1D).

We newly prepared the SCF^{cdc4} complex and examined the effect of Lag2 on Cdc34 binding to Cdc53. We observed that Lag2 inhibited Cdc34 binding to both rubylated and unrubylated Cdc53 *in vitro* (Figure 7A). This result might contradict with the *in vivo* data (Figure 1C, E, G), which demonstrated that Lag2 only interacted with unrubylated Cdc53. However, in the binding experiment *in vitro* (Figure 1D), we observed that Lag2 bound to small fraction of rubylated Cdc53, in addition to large amount of unrubylated Cdc53, which indicated that Lag2 had weak binding affinity with rubylated Cdc53 *in vitro*. Based on this observation, we think that Lag2 inhibits Cdc34 binding to both rubylated and unrubylated Cdc53 *in vitro*. We have clarified this point in the revised manuscript (page 21, line 16 - page 22, line 12).

2. Fig. 7: The authors need to show the effect of Lag2 on Rbx1 binding to Cdc53

We examined whether Lag2 affected Rbx1 binding to Cdc53 or not. As shown in Figure 7B, the association of Rbx1 to Cdc53 did not change with the increasing amount of recombinant Lag2. We have clarified this point in the revised manuscript (page 22, lines 14 - 17).

3. Reduced Aah1 steady state levels are not proof for increased protein degradation. At a minimum the RNA data need to be included. Similar the Sic1 experiment wasn't done in a way that unambiguously measures Sic1 degradation. Sic is quite stable in many phases of the cell cycle. Sic1 degradation should be measured in cells released from an alpha-factor synchrony combined with GAL-shut-off. I also think that Cln1 or Cln2 degradation needs to be analyzed, because their degradation is not affected by cell cycle position and can be easily measured.

We examined the abundance of mRNA of Aah1 in detected yeast stains and found they were in the similar levels (Figure 6E). Next, we further investigated the turnover of Sic1 after synchronized by

α-factor (Figure 6F). The deletion of *lag2* induced a decrease of the stability of Sic1, compared to that in wild-type cells. Next, we compared the stability of Cln2 in asynchoronized cells. Similarly, a deduced stability of Cln2 was observed in the *lag2* deleted yeast stain. We have clarified this point in the revised manuscript (page 20, line 14 - page 21, line 13).

4. Fig. 4B and C: I still think it is not adequate to express the growth defect as "relative colony size". Growth curves should be shown instead. The reduced colony size could also, for example, be a reflection of delayed recovery from the liquid-to-solid medium shift. In addition, it is important to include a rub1 lag2 dcn1 triple mutant to test whether the growth defect depends on rubylation.

To rule out the possibility that the reduced colony size was caused by delayed recovery from the liquid-to-solid medium shift, we further measured their growth rates in YPD medium (Figure 4D). Consistent with the previous results (Figure 4A, B, C), double deletions of *lag2* and *dcn1* inhibit the cell growth. Because loss of Dcn1 function suppresses the rubylation to Cdc53, we wondered how the combinational deletions of *rub1*, *lag2* and *dcn1* influence the cell growth. Single deletion of *rub1* did not show clear growth effect. However, double deletion of *lag2* and *rub1* displayed similar growth defect to that of double deletion of *lag2* and *dcn1*. Triple deletion of *lag2*, *dcn1* and *rub1* did not show further growth effect, compared to the double deletion of *lag2* and *rub1*. Additional deletion of *jab1* in this triple mutant failed to affect the cell growth (data not shown). These results imply that the loss of Lag2 function, together with the defect of rubylation to Cdc53 results in the retardation of cell growth. We have clarified this point in the revised manuscript (page 17, line 13 - page 18, line 4).

Minor comments:
1. Page 10: "...Lag2 bound to all the components of SCF complex...". I think this is somewhat misleading because the experiment cannot distinguish between binding to one single component and binding to several components.

We agreed with this concern because we cannot distinguish between binding to one single component and binding to several components. We changed the text to “Lag2 bound to SCF complex”. We have clarified this point in the revised manuscript (page 10, lines 8-9).

2. Page 12: "Overexpression of Lag2 dramatically increased...(Fig. 2A)." This is overstated, there is a minimal effect.

According to the referee`s comment, we deleted the word of “dramatically”. We have clarified this point in the revised manuscript (page 12, lines 6 - 7).

3rd Editorial Decision 12 August 2009

Many thanks for submitting the revised version of your manuscript EMBOJ-200-71502R. It has now been seen again by referee 2, whose comments are appended below. As you will see, he/she finds that you have significantly improved the manuscript, but still has a number of concerns - particularly regarding the data presented in Figure 7. However, given the amount of work you have already done in improving the manuscript, and as we recognise the complexity of the interactions you are investigating, we are willing to accept your study without further major revision. I assume you are aware of the EMBO Journal Transparent Editorial Process initiative, and we hope that, with the publication of the Review Process File alongside the manuscript, the unresolved questions will be clearly visible to the readers, and will spark further investigation of these issues.

Before we can formally accept your manuscript, however, I would ask for a number of minor modifications to the text, as suggested by the referee. Taking each point in turn:

1) If you have quantitation on the data presented in Figure 7A, this would clearly be valuable. Otherwise, we will not insist on such quantitation, since the inhibition of the interaction is clear, even if variable, in both versions of the figure. It might, however, be worth mentioning this variability.
2) The question of the discrepancy between the data presented in figures 1 and 7 regarding the effects of Lag2 on rubylated versus unrubylated Cdc53 is clearly an outstanding issue. However, I (and the referee) can see that there may be a number of explanations for this, and since you explicitly mention this in the text, I am satisfied that you have addressed the point as clearly as possible at this stage.
3) I leave it up to you whether to remove the data in figure 7B or to keep it in. However, if you do keep it, I would ask that you make it clearer in the text that you are assaying the ability of Lag2 to dissociate Rbx1/Cdc53, rather than its ability to inhibit the association.
4) The referee criticises the use of coomassie stains as loading controls. I agree that this is not optimal. If you did run more appropriate loading controls, it would be useful if you could modify the figure accordingly, but again, we will not insist on this at this late stage.
5) Please could you go through the text and correct outstanding grammatical errors as pointed out by the referee?

From points 1 and 4, it is possible that you may want to modify the figures. If so, we will have to do this as a formal revision. If, however, you will only be making changes to the text, the easiest way to do this would be for you to e-mail me with a revised .doc file that we will upload in place of the original version. Please just let me know how you would like to proceed.

REFEREE REPORTS

Referee 2 comments:
The authors addressed most of my concerns (except one, see below) and the results of the new experiments are consistent with their model. The one exception relates to the experiments addressing a role of Lag2 in Cdc34 binding to SCF (Fig. 7). In my opinion, this is a crucial part of the manuscript and the authors make quite a strong statement in the abstract ("Lag2 negatively controls the ubiquitylation activities of SCF E3 ligase by interrupting the association of Cdc34 to SCF complex"), which I am not convinced is supported by the data. I believe that the genetic results as well as the degradation data are consistent with this idea but I am concerned about the biochemical data (Fig. 7).

i) I am somewhat concerned about the reproducibility of these binding experiments. The previous version of Fig. 7 showed a substantial inhibition of Cdc34-SCF association in the presence of Lag2 (it was obvious at 100ng Lag2 and almost complete at 300ng). The new experiment shows a much more subtle effect of Lag2 (on the rubylated form of Cdc53). Saturation of the signal might be a component here. Some sort of quantitation could resolve this issue.

ii) The main concern however is that the effect is at least as strong, if not stronger, on the rubylated form of Cdc53 as compared to the de-rubylated Cdc53. This contradicts the main thesis of the manuscript demonstrating that Lag2 specifically binds de-rubylated Cdc53! The authors recognize that and comment on this apparent contradiction by noting that Lag2 does bind a small fraction of rubylated Cdc53 in IP experiments. However, if Lag2 binding to Cdc53 is in any way connected to blocking Cdc34 recruitment then the dramatically lower binding of Lag2 to rubylated Cdc53 should be reflected in the Cdc34 binding assay shown in Fig. 7. I believe there are several possible explanations. To name only two, F-box mediated dimerization of rubylated and de-rubylated SCF complexes could mask the effect, or Lag2 could bind Cdc34 and interfere with interaction.

iii) Fig 7B: In my previous review I suggested to test the effect of Lag2 on Rbx1 binding to SCF. The authors present the experiment in Fig. 7B as an approach to test the effect of Lag2 on association of Rbx1 and Cdc53. However, the experiment really analyzes whether addition of Lag2 can dissociate Rbx1 from Cdc53, because the authors add Lag2 to preformed Rbx1/Cdc53 complexes. I think in the current form this experiment does not add anything to the manuscript unless the authors can demonstrate that Lag2 can dissociate cdc34 from a Cdc34/SCF complex.

Other comments:

1) Fig. 6: I think that showing coomassie stains as loading controls is not very meaningful, particularly if the stain is as saturated as in the panel shown. I leave this up to the editor, but I think the results would be more convincing with a proper loading control (actin or something else) detected by immunostaining.
2) the new sections are hard to read and have many gramatical errors. The authors should fix that. I.e. pages 20 and 21: "deduce" should be "reduce", or page 20:
.."cultured with YPD medium, harvested to 6 OD600, lysed...." should be "cultured in YPD medium to an OD600=6, lysed..."

2nd Revision - Authors' Response 13 August 2009

Response to Editorial Comments:

1) If you have quantitation on the data presented in Figure 7A, this would clearly be valuable. Otherwise, we will not insist on such quantitation, since the inhibition of the interaction is clear, even if variable, in both versions of the figure. It might, however, be worth mentioning this variability.

We have not quantified the data presented in Figure 7A, so, we do not show the quantitaion. In the updated text, we add the sentence "In our study, Lag2 reproducibly suppressed the binding of Cdc34 to SCFcdc4, even though the inhibiting efficiency displayed some kind of variability." (Page 22, lines 6-8)

2) The question of the discrepancy between the data presented in figures 1 and 7 regarding the effects of Lag2 on rubylated versus unrubylated Cdc53 is clearly an outstanding issue. However, I (and the referee) can see that there may be a number of explanations for this, and since you explicitly mention this in the text, I am satisfied that you have addressed the point as clearly as possible at this stage.

At this stage, we appreciated the comments of editor. We will further investigate this issue in the followed work.

3) I leave it up to you whether to remove the data in figure 7B or to keep it in. However, if you do keep it, I would ask that you make it clearer in the text that you are assaying the ability of Lag2 to dissociate Rbx1/Cdc53, rather than its ability to inhibit the association.

We would like to keep the data in Figure 7B and we modified the text as editor suggested. "Finally, we examined whether Lag2 dissociated Rbx1 from Cdc53 in vitro. As shown in Figure 7B, the increasing amount of recombinant Lag2 did not induce the dissociation of Rbx1 from Cdc53." (Page 22, line 17-page 23, line 1)

4) The referee criticises the use of coomassie stains as loading controls. I agree that this is not optimal. If you did run more appropriate loading controls, it would be useful if you could modify the figure accordingly, but again, we will not insist on this at this late stage.

We would like to keep using coomassie stains as loading controls, because in the former submission step, we also showed coomassie stains as loading controls in the experiments of protein stability.

5) Please could you go through the text and correct outstanding grammatical errors as pointed out by the referee?

We already corrected the grammatical errors as referee pointed out:
pages 20 and 21: "deduce" to "reduce";
all the expressions of OD600 .

Additionally, we deleted the sentence as followed in Plasmid construction, Materials and methods session, "DNAs encoding wild-type or mutant versions of Saccharomyces cerevisiae Cdc53 tagged

at their N-termini with three copies of the FLAG epitope (3FLAG) were subcloned into the p416ADH vector", because in the resubmission step, we did not show the data using these plasmids. (71502.doc page 30, lines 7-9)

